



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|--|-----------|---|
| (51) International Patent Classification 5 : A61K 39/395, 37/02 | A1 | (11) International Publication Number: WO 93/19783 (43) International Publication Date: 14 October 1993 (14.10.93) |
| (21) International Application Number: PCT/US93/03068 (22) International Filing Date: 1 April 1993 (01.04.93) (30) Priority data: 07/860,704 1 April 1992 (01.04.92) US (71) Applicant: THE WHITTIER INSTITUTE FOR DIA- BETES AND ENDOCRINOLOGY [US/US]; 9894 Genesee Avenue, La Jolla, CA 92037 (US). (72) Inventors: LOGAN, Ann ; Philomal Cottage, Sytchamp- ton, Stourport-on-Severn, Worcestershire DY13 9TA (GB). BAIRD, Andrew ; 5039 Via Papel, San Diego, CA 92122 (US). | | (74) Agents: CAMPBELL, Cathryn et al.; Campbell & Flores, 4370 La Jolla Village Drive, Suite 700, San Diego, CA 92122 (US). (81) Designated States: AU, BB, BG, BR, CA, CZ, FI, HU, JP, KP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> |
| (54) Title: METHODS OF INHIBITING OR ENHANCING SCAR FORMATION IN THE CNS (57) Abstract <p>The present invention relates to methods for preventing, suppressing or treating a CNS pathology characterized by a deleterious accumulation of extracellular matrix in a tissue by contacting the tissue with an agent that inhibits the extracellular matrix producing activity of TGF-β. The methods can be used to prevent, suppress or treat scar formation in the CNS. Agents that are useful in the present methods include neutralizing anti-TGF-β antibodies, Arg-Gly-Asp-containing peptides, decorin and its functional equivalents such as biglycan, and TGF-β antagonists. The present invention further provides methods for preventing, suppressing or treating a CNS pathology characterized by the insufficient accumulation of extracellular matrix. Agents that enhance the production of extracellular matrix, such as TGF-β, can be used in such methods. Finally, the present invention provides pharmaceutical compositions containing these agents, which can be administered to patients to inhibit or enhance the production of extracellular matrix in the CNS.</p> | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria | FR | France | MR | Mauritania |
| AU | Australia | GA | Gabon | MW | Malawi |
| BB | Barbados | GB | United Kingdom | NL | Netherlands |
| BE | Belgium | GN | Guinea | NO | Norway |
| BF | Burkina Faso | GR | Greece | NZ | New Zealand |
| BG | Bulgaria | HU | Hungary | PL | Poland |
| BJ | Benin | IE | Ireland | PT | Portugal |
| BR | Brazil | IT | Italy | RO | Romania |
| CA | Canada | JP | Japan | RU | Russian Federation |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SU | Sudan |
| CG | Congo | KR | Republic of Korea | SE | Sweden |
| CH | Switzerland | KZ | Kazakhstan | SK | Slovak Republic |
| CI | Côte d'Ivoire | LJ | Liechtenstein | SN | Senegal |
| CM | Cameroon | LK | Sri Lanka | SU | Soviet Union |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | MC | Monaco | TG | Togo |
| DE | Germany | MG | Madagascar | UA | Ukraine |
| DK | Denmark | ML | Mali | US | United States of America |
| ES | Spain | MN | Mongolia | VN | Viet Nam |
| FI | Finland | | | | |

1
METHODS OF INHIBITING OR ENHANCING
SCAR FORMATION IN THE CNS

The present invention was supported in part by Grant No. DK 18811 and NS-28121 from National Institute of Health. The Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

This invention relates generally to growth factors and more specifically, to the influence of transforming growth factor- β (TGF- β) on scar formation and extracellular matrix production in the central nervous system (CNS).

Complete lesions of neural pathways in the adult mammalian CNS are rarely followed by significant functional recovery. After a penetrating injury of the brain or spinal cord, a complex sequence of tissue-specific cellular events is initiated, including a general inflammatory response, angiogenesis, widespread reactive gliosis and the formation of a dense permanent scar of mesodermal origin. These responses are accompanied by transient neuronal sprouting and synaptogenesis, but in most cases the growth responses of neurons are aborted as the glial/meningeal scar becomes organized as discussed in Maxwell et al., Phil. Trans. R. Soc. Lond. 328:479-499 (1990).

There are many theories to explain the failure of axonal growth after injury to the CNS. They attribute the failure to an absence of trophic cues such as growth factors (Logan, Brit. J. Hosp. Med. 43:428-437 (1990)) or to the release of growth inhibitory substances (Schnell & Schwab, Nature 343:269-272 (1990)). The mature scar, with its dense fibrous connective tissue bordered by an astrocytic glia limitans, is a physical barrier to axonal growth. It may be that deficiencies in the extracellular environment of the growing neurites restrict their growth

so that they reach the scar tissue after the barrier is formed. Axonal penetration through scar tissue does not occur in the CNS.

Various pathologies are characterized by a deleterious accumulation of extracellular matrix materials. For example, in progressive glomerular disease, extracellular matrix accumulates in the mesangium or along the glomerular basement membrane, eventually causing end-stage disease and uremia. Similarly, adult or acute respiratory distress syndrome (ARDS) involves the accumulation of matrix materials in the lung, while cirrhosis of the liver is characterized by deleterious matrix accumulation evidenced by scarring in the liver.

At present, there are no therapies available to promote successful regeneration and functional reconnection of damaged neural pathways. Any clinical paradigm designed to promote regeneration of central neural pathways must include a regime for reduction of extracellular matrix deposition at the wound site.

Thus, a need exists to determine the factors that regulate accumulation of matrix components in the CNS after injury. A need also exists to control such factors to prevent, limit or treat pathogenic conditions characterized by inappropriate extracellular matrix formation in the CNS. The present invention satisfies these needs and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention relates to methods for preventing, suppressing or treating a CNS pathology characterized by a deleterious accumulation of extracellular matrix in a tissue by contacting the tissue with an agent that inhibits the extracellular matrix.

producing activity of TGF- β . The methods can be used to prevent, suppress or treat scar formation in the CNS.

Agents that are useful in the present methods include, for example, neutralizing anti-TGF- β antibodies, Arg-Gly-Asp-containing peptides, decorin and its functional equivalents such as biglycan. Additionally, such agents can also be TGF- β antagonists that compete with TGF- β in binding to a TGF- β receptor. Pharmaceutical compositions containing these agents can be administered to the patients to inhibit the activity of TGF- β 1 in the CNS.

The present invention further relates to methods for preventing, suppressing or treating a CNS pathology characterized by an insufficient accumulation of extracellular matrix by contacting a tissue with an agent that promotes extracellular matrix formation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention generally relates to CNS injuries, and more particularly the presence of TGF- β 1 in injured CNS tissues. Although mRNA for TGF- β 1, TGF- β 2 and TGF- β 3 are detectable in embryonic mouse brain, only TGF- β 2 and TGF- β 3 have been localized in unlesioned adult rat brain. In the experiments described below, very low levels of TGF- β 1 expression were detected in the unlesioned adult rat brain both by in situ hybridization and immunostaining. This observation is in agreement with the results described in Miller et al., Molec. Endocrinol. 3:1926-1934 (1989) and Wilcox & Derynck, Mol. Cell. Biol. 8:3415-3422 (1988), in which TGF- β 1 expression was not detected in adult mouse brain.

A number of growth factors are known to mediate various injury responses in peripheral tissues. In

particular, TGF- β is a potent stimulator of extracellular matrix deposition in peripheral tissue wounds. TGF- β has a profound influence on extracellular matrix production, including increasing collagen, fibronectin, and
5 proteoglycan expression. This growth factor also increases integrin expression, decreases the synthesis of proteases which degrade extracellular matrix components such as collagenase and transin, and increases the expression of protease inhibitors, such as the plasminogen activator
10 inhibitor type 1 and the tissue specific inhibitor of metalloprotease.

TGF- β is a multifunctional cytokine that plays an important role in regulating repair and regeneration following tissue injury. Three isoforms of TGF- β , TGF- β 1,
15 2, and 3, are expressed in mammals and to date show similar properties in vitro. Platelets contain high concentrations of TGF- β , and upon degranulation at a site of injury, release TGF- β into the surrounding tissue. TGF- β then initiates a sequence of events that promotes healing
20 including (1) chemoattraction of monocytes, neutrophils, and fibroblasts, (2) autoinduction of TGF- β production and stimulation of monocytes to secrete interleukin-1 (IL-1), tumor necrosis factor and other cytokines, (3) induction of angiogenesis and cell proliferation, (4) control of
25 inflammation and cell toxicity by acting as a potent immunosuppressant and inhibitor of peroxide release, and (5) increased deposition of extracellular matrix.

The effect of TGF- β on extracellular matrix is a key feature of its functional activities. TGF- β stimulates
30 the synthesis of individual matrix components such as fibronectin, collagens and proteoglycans and simultaneously blocks matrix degradation by decreasing the synthesis of proteases and increasing the levels of protease inhibitors as described in Edwards et al., EMBO 6:1899 (1987) and
35 Laiho et al., J. Biol. Chem. 262:17467 (1987). TGF- β also

increases the expression of integrins and changes their relative proportions on the surface of cells in a manner that could facilitate adhesion to matrix as reported in Ignatz & Massague, Cell 51:189 (1987).

- 5 The mature form of TGF- β is comprised of two identical chains, each of 112 amino acids. The amino acid sequence of TGF- β is as follows:

Ala Leu Asp Thr Asn Tyr Cys Phe Ser Ser Thr Glu Lys Asn Cys
Cys Val Arg Gln Leu Tyr Ile Asp Phe Arg Lys Asp Leu Gly Trp
10 Lys Trp Ile His Glu Pro Lys Gly Tyr His Ala Asn Phe Cys Leu
Gly Pro Cys Pro Tyr Ile Trp Ser Leu Asp Thr Gln Tyr Ser Lys
Val Leu Ala Leu Tyr Asn Gln His Asn Pro Gly Ala Ser Ala Ala
Pro Cys Cys Val Pro Gln Ala Leu Glu Pro Leu Pro Ile Val Tyr
Tyr Val Gly Arg Lys Pro Lys Val Glu Gln Leu Ser Asn Met Ile
15 Val Arg Ser Cys Lys Cys Ser. (SEQ. ID. NO. 1)

- In peripheral tissues TGF- β is a potent stimulant for scar tissue formation characterized by the excessive accumulation of extracellular matrix components. Various agents have been found that block the stimulatory effect of
20 TGF- β on proteoglycan production resulting in reduced extracellular matrix formation in peripheral tissues as described, for example, in Ruoslahti and Yamaguchi, Cell 64:867-869 (1991). Such agents include, for example, anti-TGF- β antibodies, RGD-containing peptides and decorin.

- 25 However, the role of TGF- β 1 in CNS wounds was not known until the discovery relating to the present invention. The focal elevation of TGF- β 1 mRNA and protein observed three days after lesion, suggests that this growth
30 factor plays a role in regulating the CNS responses to injury. The association of TGF- β 1 mRNA and protein with injury-responsive cells such as glia, neurons and vascular endothelial cells, indicates that the factor is

endogenously produced, and not simply supplied to the wound via blood platelets. The observations made in this model agree with those obtained by others who have also demonstrated increased TGF- β 1 mRNA in rat hippocampus after entorhinal cortex lesion by northern blot hybridization (Nicols et al., Neurosci. Res. 28:134-139 (1991)) and by in situ hybridization in rat cerebral cortex, striatum and hippocampus after a hypoxic-ischemic insult (Klempt et al., Proc. 73rd Ann. Meeting Am. Endocrin. Soc. p. 1809 (Abstract 1991)). The results are particularly surprising because the presence of TGF- β 1 in injured CNS tissue was believed to be a result of a compromised blood brain barrier. However, in the studies described below, it has been shown that the blood brain barrier is regenerated before scarring occurs.

The in vivo infusion studies detailed in the examples below further evidence that TGF- β 1 is a prime regulator of matrix production in the CNS after injury. A dramatic increase in fibrous matrix material deposited within the wound is observed in all animals infused with recombinant TGF- β 1. Thus, in the injured CNS, as in peripheral tissues, TGF- β 1 enhances matrix deposition and therefore promotes fibrous scar formation. The reactive gliosis that occurs in the neuropile surrounding the wound is apparently unaffected by the treatment as is astrocyte association into a glial membrane, suggesting that, for this part of the response at least, TGF- β 1 is not a limiting trophic factor. The observed dramatic increase in number of cells of the macrophage/microglial lineage in the injured neuropile of TGF- β 1 infused animals suggests an additional role in injury for TGF- β 1, as a chemoattractant for blood-derived cells that themselves produce multiple trophic factors.

That TGF- β 1 is produced by cells in the damaged tissue suggests that it is acting as an endogenous

stimulant of scarring responses in the CNS and, in particular, of matrix deposition. Immunoneutralization of endogenous TGF- β 1 activity results, in some cases, in a dramatic reduction in matrix deposition in the CNS wound, thus establishing an intrinsic activity for endogenous TGF- β 1. The results suggest that a reduction of the dense permanent scar that is deposited at the site of injury, and which blocks the path of regenerating neurons, is one step towards achieving functional reconnection of damaged neural pathways to their target organs.

The reduction in macrophage/microglial cell number in the immunoneutralized wound and the lack of an organized glial limiting membrane, despite the presence of a clear reactive gliosis response, again suggests that TGF- β 1 is exerting multiple effects in these damaged tissues. Thus, endogenous TGF- β 1 may act as a chemoattractant for blood-derived cells and for activated astrocytes, as well as a potent desmoplastic agent, promoting matrix deposition by the invading fibroblasts. The functional consequences of the immunoneutralizing effects reported here are at present unknown, but in this study we could see no evidence of active nerve regeneration (assessed using GAP 43 and RT 97 antibodies as markers of regenerating axons) in any of the treated animals after fourteen days. Presumably, additional neurotrophic agents may be required to sustain this aspect of the injury response.

In this study, although the response to infused recombinant TGF- β 1 was consistent in all animals, the response to TGF- β 1 immunoneutralization was consistently more variable. This variability may be attributed to the effective concentration and bioactivity of the infused antibody within the target tissue and to potential compensatory mechanisms of other trophic factors which may be contributing to the regulation of the cellular responses examined.

The infusion experiments described below are the first to directly address the question of whether scar production in the CNS is amenable to modulation in vivo. While the modification of the injury response has been achieved using TGF- β 1-related molecules, immunoneutralization of TGF- β 1 in this model suggests that the course of scar production can be changed, particularly reduced or prevented. The development of novel therapies based on the manipulation of growth factor bioavailability within the wound in the acute phase of the injury response is a novel strategy.

The results of the studies relating to the present invention clearly implicate TGF- β 1 as a regulator of scar production after a penetrating injury to the brain or spinal cord. Furthermore, because this scar formation can preclude neuronal recovery, the results indicate a potential use for TGF- β 1 antagonists as an adjunct to those therapies designed to promote regeneration and reconnection of damaged neural pathways.

Accordingly, the present invention provides a method for preventing, suppressing or treating CNS pathologies characterized by a deleterious accumulation of extracellular matrix in a tissue. Such methods can be accomplished by contacting the tissue with an agent that inhibits the extracellular matrix producing activity of TGF- β 1. The agent can be, for example, a neutralizing anti-TGF- β antibody or a functional fragment, or an Arg-Gly-Asp-containing peptide. Preferably, such an Arg-Gly-Asp-containing peptide is between 4 and 50 amino acids in length.

Additionally, agents that act as TGF- β antagonists, such as fragments of TGF- β having sequences that bind to a TGF- β receptor, can be used in the present methods. Such antagonists should not induce the production

of extracellular matrix, but will competitively bind to such receptors to prevent TGF- β binding.

The agent can also be decorin or its functional equivalent. As used herein, "decorin" refers to a
5 proteoglycan having substantially the structural characteristics attributed to it in Krusius and Ruoslahti, PNAS (USA) 83:7638 (1986). Human fibroblast decorin has substantially the amino acid sequence presented in Krusius and Ruoslahti, supra. "Decorin" refers both to the native
10 composition and to modifications thereof which substantially retain the functional characteristics. Decorin core protein refers to decorin that no longer is substantially substituted with glycosaminoglycan and is included in the definition of decorin. Decorin can be
15 rendered glycosaminoglycan-free by enzymatic treatment, mutation or other means, such as by producing recombinant decorin in cells incapable of attaching glycosaminoglycan chains to a core protein or by synthesizing the core protein, all by means well known in the art.

20 Functional equivalents of decorin include modifications of decorin that retain its functional characteristics and molecules that are homologous to decorin, such as biglycan and fibromodulin, for example, that have the similar functional activity of decorin.
25 Modifications can include, for example, the addition of one or more side chains that do not interfere with the functional activity of the decorin core protein.

The agents useful in the methods can be obtained by purifying the native protein or by proteolytic digestion
30 of such proteins to obtain functionally active fragments according to methods known in the art. Alternatively, such agents can be synthesized or produced recombinantly by methods known in the art.

The present invention further provides methods for preventing, suppressing or treating a CNS pathology characterized by the insufficient accumulation of extracellular matrix components to promote scar formation.

5 These methods can be accomplished by administering TGF- β or a functional fragment that promotes extracellular matrix production to a patient in need of such therapy. Such methods can be used when inadequate scar formation can or does result in a CNS pathology.

10 Pharmaceutical compositions containing agents that inhibit the activity of TGF- β or increase the concentration of TGF- β and a pharmaceutically acceptable carrier can be administered to a patient to prevent, enhance or otherwise treat CNS scar formation. Suitable
15 pharmaceutically acceptable carriers include, for example, hyaluronic acid and aqueous solutions such as bicarbonate buffers, phosphate buffers, Ringer's solution and physiological saline supplemented with 5% dextrose or human serum albumin, if desired. Other pharmaceutical carriers
20 known to those skilled in the art are also contemplated. The pharmaceutical compositions can also include other reagents that are useful for the prevention or treatment of the various CNS pathologies characterized or associated with the accumulation of extracellular matrix. The dosage
25 of such pharmaceutical compositions can be readily determined by those skilled in the art based on various factors such as, for example, the type and extent of the injury, the age of the patient and the agent used.

The present invention further relates to methods
30 of detecting the presence of various CNS pathologies of a tissue characterized by an excessive accumulation of extracellular matrix components by determining the level of TGF- β 1 in the tissue. Such detection methods can be accomplished by the procedures described in the examples
35 below or by other methods known in the art.

The following examples are intended to illustrate but not limit the invention.

EXAMPLE I

Materials

5 Unless specified, all reagents were analytical grade from Sigma Chemical Co. Ltd. (St. Louis, MO) or Poole (United Kingdom). Radioisotopes were obtained from Amersham International (Arlington Heights, IL). Recombinant human TGF- β 1 was obtained from R and D Systems
10 (Minneapolis, MN). Two anti-TGF- β 1 antibodies were used in this study. The inactivating antibody used in the infusion experiments was raised in the turkey against intact native human TGF- β 1. A different antibody was used for
15 histochemistry, which was raised in the rabbit against amino acids 1-30 of human TGF- β 1. Both of the anti-TGF- β 1 antibodies were prepared and characterized by M.B. Sporn and K.C. Flanders (National Institutes of Health, Bethesda, MD) and described in Logan et al., supra.

EXAMPLE II

Animals and Surgery

20 Surgical and animal care procedures were carried out with strict adherence to the guidelines set out in the "NIH guide for the care and use of laboratory animals," National Institutes of Health Publications No. 80-23.
25 Groups of adult, female Sprague-Dawley rats (250 grams) were used in the study. Animals were anaesthetized with an intraperitoneal injection of a mixture of acepromazine (1.875 mg/kg), ketamine (3.75 mg/kg) and xylazine (1.9 mg/kg). Following craniotomy, a stereotactically defined,
30 4mm deep, rostro-caudal knife-wound incision was made vertically into the right occipital cortex, corpus callosum and presubiculum, placed 1 mm anterior to Bregma/1.4 mm lateral of the mid-line, so it penetrated the anterior

lateral ventricle at some point in its length. Control animals underwent craniotomy but no lesion was made.

Three days after surgery, four lesioned and two control rats were put under deep anaesthesia with the same anaesthetic and perfused transcardially with 300 ml of 0.9% (wt/vol) saline, 250 ml of 4% (wt/vol) paraformaldehyde (PFA) in 0.1 M acetate buffer, pH 6.5, followed by 500 ml of 4% (wt/vol) PFA plus 0.05% (wt/vol) glutaraldehyde in 0.1 M borate buffer, pH 9.5, using the pH shift method described in Simmons et al., J. Histotechnology 12:169-181 (1989), incorporated herein by reference. The brains were removed and post-fixed overnight at 4°C in 4% (wt/vol) PFA in 0.1 M borate buffer containing 10% (wt/vol) sucrose. These brains were processed for in situ hybridization and immunoperoxidase staining to reveal expression of TGF- β 1 mRNA and protein in the tissue surrounding the lesion.

The remaining three groups of six animals underwent a further surgical procedure at the same time as the placement of the lesion. In these animals, a vertical stainless steel cannula was inserted through the cranium into the posterior of the right lateral ventricle. The cannula was cemented into place with a dental cement platform which was stabilized by three stainless steel machine screws inserted into the cranium distal to the site of cannulation. The cannula was attached under the skin, via a flexible vinyl catheter, to a ready-primed Alzet mini-osmotic pump (model 2002, Alza Corporation, Palo Alto, CA) which was inserted into a sub-cutaneous pouch made in the dorsal neck region of the animal. The pumps supplied test agents to the cannula at a prescribed rate (0.5 μ l/hr) and dose over a 14 day period.

In the main experiment the mini-osmotic pumps were primed to supply a basic infusion vehicle of phosphate-buffered artificial cerebrospinal fluid: 150 mM

NaCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 2.0 mM K₂HPO₄, 10.0 mM glucose, pH 7.4. In addition, 0.1% autologous rat serum was included in the vehicle to reduce absorption losses within the infusion apparatus. TGF-β1 protein and anti-TGF-β1 antiserum were diluted to their appropriate concentrations directly into this vehicle. In vitro experiments to monitor the stability of the infused TGF-β1 and anti-TGF-β1 antibody indicated that biological activity of both protein and antibody is preserved at 37°C over a 14 day period. Six animals were infused with vehicle containing a 1:100 dilution of non-immune turkey serum (controls), six animals with vehicle containing a 1:100 dilution of turkey anti-TGF-β1 antiserum raised in turkeys against native human TGF-β1, and six animals with vehicle containing 150 ng/ml recombinant human TGF-β1. Thus, the TGF-β1-infused animals received 1.8 ng/day recombinant human TGF-β1 and the antiserum-infused animals received 0.12 μl/day non-immune or anti-TGF-β1 turkey antiserum. Fourteen days after cannula implantation and continuous delivery of experimental infusion solutions, all animals were processed as previously described and histochemical evaluation of the lesion site was performed by immunofluorescent staining.

EXAMPLE III

In Situ Hybridization of TGF-β1 mRNA

In situ hybridization of TGF-β1 mRNA used the Hind III-Xba I fragment of 0.985 kbp, derived from the major coding region of the rat TGF-β1 precursor (Qian et al., Nucl. Acids Res. 18:3059 (1990)), which was sub-cloned into pBluescript SK+ (Stratagene, San Diego, CA). The antisense RNA strand of the coding sequence was transcribed using T7 polymerase and ³⁵S-UTP according to manufacturer's instructions. ³⁵S-UTP labelled RNA probes encoding sense strands of 5' non-coding sequences were prepared with T3 RNA polymerase and used for alternate control tissue

sections.

The fixed brains were frozen in O.C.T. compound (Miles Laboratories Inc., Naperville, IL) in dry ice and stored at -80°C. At a later date, 20 µm frozen sections were cut and collected in cryoprotectant solution (50% 0.05 M sodium phosphate buffer, pH 7.3, 30% ethylene glycol and 20% glycerol) and stored at -20°C. Subsequently cryoprotected brain sections were washed thoroughly in phosphate-buffered saline (PBS), mounted on poly-L-lysine coated slides, dried under vacuum and stored at -80°C until use.

For analysis, sections were digested with 10 µg/ml of proteinase K in 0.1 M Tris (pH 8.0) containing 50 mM EDTA at 37°C for 30 minutes and then rinsed in deionized water, followed by an incubation in 0.1 M triethanolamine hydrochloride (TEA), pH 8.0 for 3 minutes. Sections were acetylated for 10 minutes with 0.25% (wt/vol) acetic anhydride in 0.1 M TEA, rinsed in 2 X SSC (prepared from a 20 X stock solution which contains 3 M NaCl and 0.015 M sodium citrate), dehydrated through a graded series of ethanol washes and then air dried for 2 hours before hybridization.

Hybridization with the labelled TGF-β1 antisense or sense probes (1 x 10⁷ cpm/ml) was performed at 55°C overnight in 10 mM Tris (pH 8.0) containing 50% (wt/vol) formamide, 0.3 M NaCl, 1 mM EDTA, 10 mM dithiothreitol (DTT), 1 x Denhardt's solution (0.1 g Ficoll 400, 0.1 g polyvinylpyrrolidone, 0.1 g of bovine serum albumin), and 10% (wt/vol) dextran sulfate. After hybridization, sections were rinsed for 1 hour in 4 X SSC and treated with 25 µg/ml of ribonuclease A in 10 mM Tris (pH 8.0) containing 0.5 M NaCl, 1 mM EDTA at 37°C for 30 minutes. This treatment was followed by increasing high stringency washes of SSC containing 1 mM DTT, followed by a final wash

in-0.1 X SSC at 65°C for 30 minutes.

Slides were then dehydrated through a graded series of ethanol, until absolute ethanol, dried under vacuum and exposed to Bmax hyperfilm (Amersham) for 5 days at 4°C to examine gross changes in mRNA. For microscopic analysis, slides were coated with Kodak NTB-2 liquid autoradiograph emulsion and exposed at 4°C for 2-3 weeks. They were developed in Kodak D-19, rinsed briefly in water, and fixed in Kodak rapid fixer. After washing in distilled water for at least 45 minutes, slides were counterstained with Harris' haematoxylin in order to visualize the cells. Silver grains were examined by dark field and bright field microscopy.

Bright and dark field views of the lesion site were taken. After three days, an intense signal was observed for TGF- β 1 mRNA in the neuropile bordering the lesion site. This focal elevation of TGF- β 1 mRNA suggests a local expression of TGF- β 1 within the damaged neural tissue in response to injury. Under higher power, the bright field view revealed that the signal was mainly associated with cells of neuronal and astrocytic visual phenotype, although signal was also seen associated with endothelial cells of the microvasculature and in the local meninges. The increased level of signal seen in the lesioned hemisphere was striking when compared to that seen in the contralateral hemisphere or in sections of unlesioned brain, which were processed identically and simultaneously. Since the signal observed in the brains of control animals was also minimal, it seems that there is normally very low expression of TGF- β 1 mRNA in this tissue. The hybridization signal observed in sections from lesioned rat brains was specific, since adjacent tissue sections hybridized with the sense strand of cRNA show no signal.

EXAMPLE IVImmunoperoxidase Staining of TGF- β 1

Immunoperoxidase staining for TGF- β 1 in 20 μ m frozen sections of brain was accomplished using the ABC Vactastain Elite kit (Vector Laboratories Ltd., Burlingame, CA) according to the manufacturer's instructions. The primary antiserum raised against TGF- β 1 has been previously described and characterized in Flanders et al., J. Cell Biol. 108:653-660 (1989). It is an IgG fraction of a rabbit polyclonal, raised against amino acids 1-30 of human TGF- β 1, which was purified by passage over a protein A-Sepharose column.

For analysis, the cryoprotected 20 μ m sections were washed thoroughly in PBS, mounted onto gelatin coated slides, rinsed again in PBS and the endogenous peroxidase was quenched by incubating the sections in 0.3% (vol/vol) hydrogen peroxide in PBS for 30 minutes. The sections were rinsed in PBS and incubated for 30 minutes in 1.5% (vol/vol) normal goat serum, diluted in PBS containing 0.3% (vol/vol) Triton X-100, in order to block non-specific binding. Following this procedure, the sections were incubated for 24 hours at 4°C with protein-A purified rabbit anti-TGF- β 1 antibody (0.015 mg/ml) diluted in PBS containing 0.3% (vol/vol) Triton X-100 and 1% (wt/vol) bovine serum albumin (BSA). They were then rinsed and incubated with a 1:200 dilution of biotinylated goat anti-rabbit IgG (Vector) for 45 minutes, rinsed and incubated with avidin-biotin-peroxidase complex (Vector) for 30 minutes. After rinsing in PBS, the sections were treated with 0.5 mg/ml of 3'3'-diaminobenzidine (DAB), diluted in PBS containing 0.01% (vol/vol) hydrogen peroxide for 5 minutes. All steps were separated by buffer washes consisting of PBS, pH 7.4, containing 0.3% (vol/vol) Triton X-100. The sections were finally washed in PBS, counterstained with Harris' haematoxylin, dehydrated,

cleared, and mounted. Sections incubated with anti-TGF- β 1 antibody pre-incubated with recombinant TGF- β 1 or without primary antibody were used as controls. Sections processed with the control procedures failed to stain.

5 The immunocytochemical localization of TGF- β 1 in the wound was determined three days after injury. The appearance of immunoreactive TGF- β 1 seen in damaged neural tissue correlates with the extent of mRNA induction observed by in situ hybridization. Under high
10 magnification, the predominant cell types (by morphological criteria) localizing strong TGF- β 1 immunoreactivity three days after injury were the astrocyte and macrophage. The staining is mostly limited to damaged neural tissues bordering the forming glia limitans and thus appears to be
15 primarily extracellular. In this study, no immunoreactivity was observed in the contralateral hemisphere or in sections of normal, unlesioned brain processed simultaneously.

EXAMPLE V

20 Immunofluorescent Staining of the Wound

After perfusion fixation, the brains of the fourteen day cannulated and continuously infused animals were washed in PBS overnight at 4°C, dehydrated in graded alcohols, embedded in polyester wax (melting point 37°C)
25 and stored at 4°C. Sections (7 μ m) were cut on a microtome fitted with a cooled chuck and floated onto a gelatin solution (10 mg/ml) on subbed slides and air-dried.

The antibodies used to identify cellular changes in the wounds were rabbit anti-bovine glial fibrillar
30 acidic protein (GFAP) as a marker of activated astrocytes, rabbit anti-mouse fibronectin to visualize matrix deposition within the wound and rabbit anti-mouse ED1 as a marker of cells of the macrophage/microglial lineage. All

of these antibodies were obtained from Dakopatt Ltd. (High Wycombe, U.K.) and were used at a dilution of 1:200 in PBS containing 1% (wt/vol) BSA.

The mounted brain sections were dewaxed, rehydrated and placed in PBS containing 0.1% (vol/vol) Tween-20 for 15 minutes. They were then incubated in the specific antibody for 1-12 hours at room temperature. After three washes in PBS, sections were incubated in goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC, Sigma), diluted to 1:100. The sections were washed in three changes of PBS and mounted in a non-quenching mountant. For controls, either the first or second antibody was omitted, all were negative. The sections were examined with an Olympus BH-2 microscope with a fluorescent attachment. Photomicrographs were taken on Ilford HP5 film, rated at 400 ISO.

EXAMPLE VI

General Appearance of the Lesion

Three days after the induced injury, oedema in the wound was still extensive. Many macrophages were present in the central lumen of the wound but macrophages and microglia were also seen in the damaged neuropile. Numerous meningeal-fibroblasts were present in the center of the wound in the superficial cortex and scar tissue was beginning to form between the cut edges of the cortical neuropile, but had not yet penetrated the depths of the lesion. Reactive astrocytes were particularly numerous at the damaged margin of the neuropile, and also extended well into the intact neuropile.

At 14 days the major cellular events are complete. Briefly, the scar tissue had contracted, bringing the cut borders of the neuropile close together. Some residual macrophages were visible in the fibrous

tissue which had been deposited at the center of the lesion site. A fully developed glia limitans was present that lines the cut margins of the neuropile and surrounds a thin core of fibrous scar tissue. Reactive astrocytes were still visible through the damaged and intact neuropile, but the gliosis was receding.

EXAMPLE VIII

Characterization of the Wound After Infusion of Recombinant TGF- β 1 and TGF- β 1 Neutralizing Antibodies

The efficacy of the infusion method for the delivery of test agents to cells at the site of cannulation and at the site of lesion was tested by infusing a 1:100 dilution of non-immune rabbit antiserum into the right lateral ventricle of two lesioned rats over a two week period (0.12 μ l antiserum/day directly into the CSF). After fixation by perfusion, the brains were processed for immunoperoxidase staining using an anti-rabbit IgG antibody (Dakopatt Ltd., High Wycombe, U.K.), to examine the extent of penetration of the infused antiserum. The results of this test show an extensive distribution of the infusate is revealed throughout the tissue surrounding both the site of cannulation and the site of lesion.

Penetration of infused recombinant protein and antiserum into the tissue surrounding the lesion site was confirmed at fourteen days in each experimental animal by immunoperoxidase staining. By this time the endogenous expression of TGF- β 1 has virtually subsided and negligible TGF- β 1 is detectable in the control, vehicle-infused animals. There was no observed effect of any of the continuous infusions into the CSF on the gross morphology of the ventricles, assessed by visual and microscopic inspection of tissue sections. The effect on the injury response of modulating TGF- β 1 availability at the wound site by continuous infusion of TGF- β 1-related molecules is

discussed in more detail below. Briefly, the TGF- β 1 infused wounds show a normal reactive gliosis response, an abnormal amount of fibronectin deposition and an increased number of macrophage and microglial cells when compared to control. The anti-TGF- β 1 antiserum infused animals show a normal reactive gliosis response, but no organization of astrocytes to form a limiting glia limitans at the margins of the damaged neuropile. There is also a complete absence of immunoreactive fibronectin within the wound and a reduced number of macrophage/microglial cells when compared to control.

A. Control, Vehicle Infusions

In rats with wounds that had been continuously infused with vehicle plus non-immune turkey IgG, immunofluorescent staining of sections after 14 days shows the scar tissue has contracted bringing together the cut borders of the neuropile. In the center of the wound was a thin layer of fibrous tissue represented by the fluorescent fibronectin, which is visible after staining with anti-fibronectin antibody. Anti-ED1 antibody, which detects cells of the macrophage/microglial lineage, revealed residual cells in the center of the wound and in neural tissues bordering the scar. GFAP-positive astrocytes were still abundant in the tissue around the wound and are particularly numerous at the lesion edge, where they associate to construct a glia limitans which helps to reform the blood-brain barrier. The scar of these animals was deemed to be normal when compared to scars seen in lesioned, non-infused rats.

B. Recombinant TGF- β 1 Infusions

Continuous infusion of the wound with 1.8 ng/day recombinant human TGF- β 1 resulted in a clear enhancement of scarring with matrix deposition being markedly increased

compared to lesioned, non-infused rats. The increase in matrix deposition was evidenced by the presence of an abnormally large area of immunoreactive fibronectin in the center of the wound, which resulted in a wide separation of the normally closely apposed cut faces of the neuropile. The fibronectin deposition was accompanied by an exactly coincident increase in collagen IV and laminin deposition. TGF- β 1 treatment also dramatically increased the number of residual macrophage/microglia cells in the neuropile, which was detected by using anti-ED 1 antibody, but had no apparent effect on the extent of reactive gliosis observed, which was visualized with anti-GFAP antibody. The continuous glia limitans, formed by the reactive astrocytes and marking the borders of the cut neuropile, was evident in these wounds.

C. Anti-TGF- β 1 Antiserum Infusions

Immunoneutralization of endogenous TGF- β 1 with 0.12 μ l/day of turkey anti-TGF- β 1 antiserum confirmed the effects of exogenous TGF- β 1 observed above. In direct contrast, this treatment markedly reduced the amount of fibrous scar tissue deposited in the wound. In this experiment, the extent of scar reduction was variable, four out of six rats responding to the treatment, with two of these showing an almost complete absence of matrix deposition in the wound. One such animal showed no apparent fibronectin deposition in the wound after fourteen days, as indicated by the absence of fibronectin immunoreactivity and a reduced residual number of ED 1-expressing macrophages/microglia. The neural tissue around this wound contained numerous reactive GFAP-positive astrocytes. However, these have not become organized into a limiting glial membrane at the margin of the lesion.

Although the invention has been described with reference to various embodiments, it should be understood

that various modifications can be made without departing from the spirit or scope of the invention. Accordingly, the invention is limited only by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: THE WHITTIER INSTITUTE FOR DIABETES AND ENDOCRINOLOGY
- 5 (ii) TITLE OF INVENTION: METHODS OF INHIBITING OR ENHANCING SCAR
FORMATION IN THE CNS
- (iii) NUMBER OF SEQUENCES: 1
- (iv) CORRESPONDENCE ADDRESS:
- 10 (A) ADDRESSEE: CAMPBELL AND FLORES
(B) STREET: 4370 LA JOLLA VILLAGE DRIVE, SUITE 700
(C) CITY: SAN DIEGO
(D) STATE: CALIFORNIA
15 (E) COUNTRY: UNITED STATES
(F) ZIP: 92122
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
20 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: PCT
(B) FILING DATE: 01-APR-1993
(C) CLASSIFICATION:
- 25 (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: CAMPBELL, CATRYN A.
(B) REGISTRATION NUMBER: 31,815
(C) REFERENCE/DOCKET NUMBER: FP-LA 9589
- (ix) TELECOMMUNICATION INFORMATION:
- 30 (A) TELEPHONE: 619-535-9001
(B) TELEFAX: 619-535-8949

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- 35 (A) LENGTH: 112 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala Leu Asp Thr Asn Tyr Cys Phe Ser Ser Thr Glu Lys Asn Cys Cys
1 5 10 15

24

Val Arg Gln Leu Tyr Ile Asp Phe Arg Lys Asp Leu Gly Trp Lys Trp
 20 25 30
 Ile His Gln Pro Lys Gly Tyr His Ala Asn Phe Cys Leu Gly Pro Cys
 35 40 45
 5 Pro Tyr Ile Trp Ser Leu Asp Thr Gln Tyr Ser Lys Val Leu Ala Leu
 50 55 60
 Tyr Asn Gln His Asn Pro Gly Ala Ser Ala Ala Pro Cys Cys Val Pro
 65 70 75 80
 10 Gln Ala Leu Gln Pro Leu Pro Ile Val Tyr Tyr Val Gly Arg Lys Pro
 85 90 95
 Lys Val Glu Gln Leu Ser Asn Met Ile Val Arg Ser Cys Lys Cys Ser
 100 105 110

We Claim:

1. A method for preventing, suppressing or treating a CNS pathology characterized by a deleterious accumulation of extracellular matrix in a tissue of the CNS, comprising contacting said tissue with an agent that inhibits the extracellular matrix producing activity of TGF- β .
2. The method of claim 1, wherein said CNS pathology is scar formation.
3. The method of claim 1, wherein said agent is a neutralizing anti-TGF- β antibody.
4. The method of claim 1, wherein said agent is an Arg-Gly-Asp-containing peptide.
5. The method of claim 1, wherein said agent is decorin.
6. The method of claim 1, wherein said agent is a functional equivalent of decorin.
7. The method of claim 7, wherein said agent is biglycan.
8. The method of claim 1, wherein said TGF- β is TGF- β 1.
9. A method for preventing, suppressing or treating a CNS pathology characterized by an insufficient accumulation of extracellular matrix in a tissue of the CNS, comprising contacting said tissue with an agent that promotes extracellular matrix production.

10. The method of claim 9, wherein said agent is TGF- β .

11. The method of claim 10, wherein said TGF- β is TGF- β 1.

5 12. The method of claim 9, wherein said agent is a functional fragment of TGF- β .

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 93/03068

| | | |
|---|--|-------------------------------------|
| I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ | | |
| According to International Patent Classification (IPC) or to both National Classification and IPC | | |
| Int.Cl. 5 A61K39/395; A61K37/02 | | |
| II. FIELDS SEARCHED | | |
| Minimum Documentation Searched ⁷ | | |
| Classification System | Classification Symbols | |
| Int.Cl. 5 | A61K | |
| Documentation Searched other than Minimum Documentation to the extent that such documents are included in the Fields Searched ⁸ | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ | | |
| Category ¹⁰ | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
| O,X | JOURNAL OF CELLULAR BIOCHEMISTRY, SUPPLEMENT 15F, 1991 (KEystone SYMPOSIA ON MOLECULAR & CELLULAR BIOLOGY, KEystone, COLORADO, US, 1-7 APRIL, 1991) 1991, NEW YORK, N.Y., US page 241 A. LOGAN ET AL. 'IN VIVO EFFECTS OF GROWTH FACTOR AGONISTS AND ANTAGONISTS ON MAMMALIAN CNS INJURY RESPONSES.' | 1-3,8-12 |
| O,Y | See Abstract No. CF 313 | 4-7 |
| Y | WO,A,9 104 748 (LA JOLLA CANCER RESEARCH FOUNDATION) 18 April 1991 see page 11, line 7 - line 12; claims 1,2,4,5-7,9 | 4 |
| <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report | |
| 12 JULY 1993 | | |
| International Searching Authority | Signature of Authorized Officer | |
| EUROPEAN PATENT OFFICE | RYCKEBOSCH A.O. | |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|---|-----------------------|
| Category* | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
| Y | WO,A,9 110 727 (LA JOLLA CANCER RESEARCH FOUNDATION) 25 July 1991 see claims 1-3,9,10,27-30 | 5-7 |
| X | WO,A,9 102 067 (MAX PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 21 February 1991 see page 53, line 1 - line 5; claims 1-3,6,9,17,58; example 8 | 8-12 |
| P,X | BRAIN RESEARCH vol. 587, no. 2, 7 August 1992, AMSTERDAM, NL pages 216 - 225 A. LOGAN ET AL. 'ENHANCED EXPRESSION OF TRANSFORMING GROWTH FACTOR BETA-1 IN THE RAT BRAIN AFTER A LOCALIZED CEREBRAL INJURY.' see page 224, left column, line 2 - line 16 see page 224, left column, line 24 - line 27 | 1 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/03068

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 1-12 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9303068
SA 72284

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

12/07/93

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| WO-A-9104748 | 18-04-91 | AU-A- 6612590 | 28-04-91 |
| | | CA-A- 2065860 | 30-03-91 |
| | | EP-A- 0494264 | 15-07-92 |
| WO-A-9110727 | 25-07-91 | None | |
| WO-A-9102067 | 21-02-91 | EP-A- 0484416 | 13-05-92 |
| | | JP-A- 5025056 | 02-02-93 |

EPO FORM 1001

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82